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Induction of Human Leukocyte Antigen (HLA)-A2-Restricted and MAGE-3-Gene-Derived Peptide-Specific Cytolytic T Lymphocytes Using Cultured Dendritic Cells From an HLA-A2 Esophageal Cancer Patient

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Background and Objectives: Using peripheral blood mononuclear cells (PBMCs) from a 10-year survivor with established human leukocyte antigen (HLA)-A2(+) and MAGE-3(+) esophageal cancer cell line (KYSE-170), we examined the induction of HLA-A2-restricted and MAGE-3-gene-derived peptide (FLWGPRALV, amino acids 271–279)-specific cytolytic T lymphocytes (CTLs).

Methods: Autologous dendritic cells (DCs) cultured with granulocyte-macrophage colony stimulating factor and interleukin-4 were used as antigen presenting cells. PBMCs were stimulated by peptide-pulsed DCs in vitro.

Results: PBMC cocultured with FLWGPRALV-pulsed DCs could induce the relevant peptide-specific CTLs, which had tumor necrosis factor production and specific cytotoxicity against relevant peptide-pulsed autologous DCs (34%, effector:target ratio = 40:1). Moreover, they showed specific cytotoxicity against the autologous esophageal cancer cell line KYSE-170 (17%, effector:target ratio = 40:1).

Conclusions: These results suggest that FLWGPRALV-pulsed cultured DCs would be a potent candidate for peptide vaccine against HLA-A2(+) and MAGE-3(+) esophageal cancer.

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Key Words: antigen presenting cell; antigenic peptide; vaccine therapy; antitumor immunity

INTRODUCTION

Human leukocyte antigen (HLA)-restricted tumor rejection antigens, including the MAGE family, have been identified in melanoma cells, other tumors, and testis [1]. HLA-restricted and antigen-derived oligopeptides also have been defined by autologous cytolytic T lympho-

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cytes (CTLs). Therefore, these antigens have presented opportunities for peptide-based vaccine therapy against human cancers. For such purposes, several peptide-specific induction methods have been examined. However, whether or not it is possible to induce cytolytic activity against autologous tumor is still unclear.

In practical application of vaccine strategies, administration of "naked" peptide (EVDPIGHLY, amino acids 161–169 [2]) encoded by MAGE-3-gene induced clinical responses in 3 of 12 HLA-A1 melanoma patients [3]. But peptide alone might induce anergy rather than effective immune response, because of lack of costimulatory signal. We had tried to use granulocyte-macrophage colony stimulating factor (GM-CSF)-cultured autologous dendritic cells (DCs) for a peptide carrier [4], since professional antigen presenting cells (APCs) play important roles in the specific immune response. Sallusto and Lanzavecchia [5] and Romani et al. [6] reported that using GM-CSF and interleukin (IL)-4 is the most efficient method of inducing DCs from peripheral blood mononuclear cells (PBMCs). We have applied this method for the in vitro induction of HLA-A2-restricted and MAGE-3-gene-derived peptide (FLWGPRALV, amino acids 271–279 [7])-specific CTLs.

In this study, we report that HLA-A2-restricted and FLWGPRALV-specific CTLs were induced using the combination of GM-CSF and IL-4-cultured DCs and PBMC from a HLA-A2(+) esophageal cancer patient and showed specific cytotoxicity against an autologous esophageal cancer cell line.

MATERIALS AND METHODS

Cell Lines

The human esophageal carcinoma cell lines KYSE-170 (MAGE-3(+); HLA-A2, A33) and KYSE-520 (MAGE-3(+); HLA-A24, 33) were established from primary tumors as previously described [8]. These cell lines were maintained in Iscove modified Dulbecco medium (IMDM; Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (FBS; Bio-Whittaker, Verviers, Belgium).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Amplification

Total ribonucleic acid (RNA) was isolated from esophageal carcinoma cell lines according to the manufacturer's instructions [9] using TRIZOL™ (Life Technologies) reagent.

Using thermal cycler (MJ Research, Inc., Watertown, MA), RT-PCR was carried out with the following primers and protocols using complementary deoxyribonucleic acid (DNA) synthesized from 100 ng of total RNA: MAGE-3 sense primer AB-1197 (5′-GGA GGA CCA GAG GCC CCC-3′), MAGE-3 antisense primer AB-913 (5′-GGA GTC CTC ATA GGA TTG GCT CC-3′) by 30 cycles (1 min at 94°C, 2 min at 70°C, 2 min at 72°C), beta-actin sense primer CHO-15 (5′-GGC ATC GTG ATG GAC TCC G-3′), beta-actin antisense primer CHO-16 (5′-GCT GGA AGG TGG ACA GCG A-3′) by 21 cycles (1 min at 94°C, 2 min at 68°C, 2 min at 72°C). Each reaction mixture (10 μl) was electrophoresed through a 1.5% agarose gel and the quality of RNA preparation was verified by PCR product to human beta-actin messenger RNA (Fig. 1).

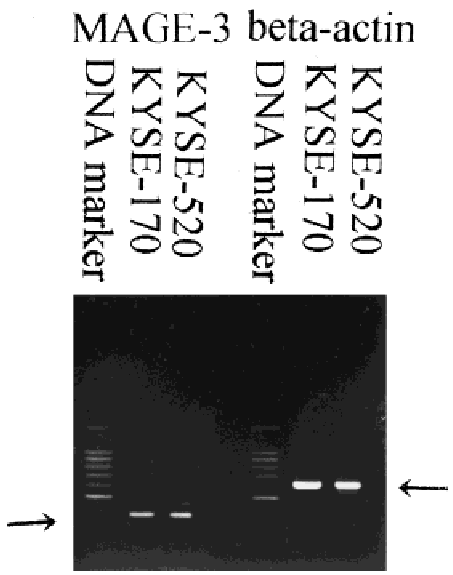

Fig. 1. Expression of MAGE-3 gene in human esophageal cancer cell lines by RT-PCR amplification. KYSE-170, KYSE-520: human esophageal cancer cell lines. Amplifications of beta-actin from the same complementary DNA samples were performed for control. The 100 bp DNA marker was purchased from Sawady Technology (Tokyo, Japan).

Peptides

HLA-A2-restricted and MAGE-3-gene-derived peptide (FLWGPRALV) and HLA-A1-restricted and MAGE-3-gene-derived peptide (EVDPIGHLY) were synthesized by Fmoc-based solid phase synthesis followed by trimethylsilylbromide protection [10]. Peptides were diluted by phosphate-buffered saline (PBS) at a concentration of 1 mg/ml and stored at −20°C until use.

Culture of DCs

PBMCs from a HLA-A2(+) patient, from whom the MAGE-3(+) esophageal cancer cell line KYSE-170 was established, were isolated on a Ficoll-Hypaque gradient and cultured for 2 hr in a 12-well plate with IMDM containing 10% FBS and then the non-adherent cells were removed by 4 gentle washings with PBS. The non-adherent cells from PBMC were cryopreserved until use. The adherent cells were cultured in AIM-V™ (Life Technologies) supplemented with 50 ng/ml GM-CSF (kindly provided by Schering-Plough, Tokyo, Japan) and

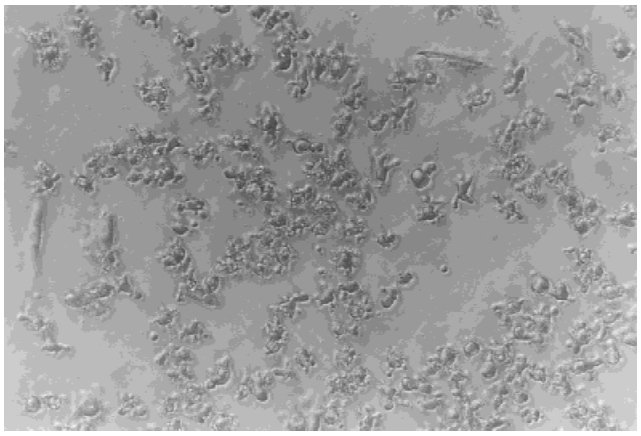

Fig. 2. Microscopic appearance of DC cultures on day 14. Phase-contrast microscopy; ×150.

500 U/ml IL-4 (kindly provided by Ono Pharmaceutical Co. Ltd., Osaka, Japan) for 2 weeks. Their characteristics were studied by microscopy and immunofluorescence staining (Fig. 2).

Induction of Effector Cells

For peptide pulsing, 14-day cultured DCs were incubated with 50 μg/ml peptide and 100 μg/ml mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan) at 37°C for 60 min in AIM-V and then washed 3 times in PBS. DCs were preincubated at 20°C overnight in the presence of 2.5 μg/ml human beta2-microglobulin (Sigma, St. Louis, MO).

The autologous non-adherent PBMCs and peptide-pulsed DCs were mixed at a ratio of 5:1 and cultured in AIM-V with 10 ng/ml IL-7 (Genzyme, Cambridge, MA). IL-2 (gracious gift by Dr. J. Hamuro, Ajinomoto Co., Inc., Tokyo, Japan) was added to the cultures at 10 U/ml on day 2. The responder cells were stimulated every 7 days with autologous peptide-pulsed DCs.

To assess the proliferation of responder cells, 1×10^5 responding cells were restimulated with 2×10^4 autologous peptide-pulsed DCs on day 21 and their viable cell numbers were measured 6 days later by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay.

For tumor necrosis factor (TNF) production assay, 1×10^5 responding cells were cocultured with 2×10^4 FLWGPRALV-pulsed DCs or EVDPIGHLY-pulsed DCs on day 28. After 24 hr culture, TNF concentration of supernatants was measured by MTT colorimetric assay using WEHI 164 clone 13 [11] and human recombinant TNF-beta (Genzyme) was used for control.

All assays were carried out in triplicate, and results are presented as mean ± standard deviation.

Immunofluorescence Analysis

These responding cells were stained by indirect immunofluorescence staining method on day 21 and counted with fluorescence microscopy. Monoclonal antibodies (mAbs) used were OKT3 (anti-CD3; American Type Culture Collection (ATCC), Rockville, MD), OKT4 (anti-CD4; ATCC), OKT8 (anti-CD8; ATCC), and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin mAbs (Boehringer Mannheim, Indianapolis, IN).

Cytolytic Assay

Standard 4 hr ^{51}Cr-release assay was performed at 5 weeks after the beginning of culture. Briefly, the target cells were radiolabeled with sodium chromate (100 μCi) for 60 min at 37°C. After labeling, they were washed 3 times and incubated for 60 min at 37°C in the presence or absence of 50 μg/ml of relevant or control peptides. After peptide pulsing, they were washed again and cocultured with responding cells for 4 hr (effector: target ratio = 40:1). After taking 100 μl of supernatants from each well, their radioactivities were counted using a gammacounter and their cytolytic activities were calculated by the following formula:

$$\% \text{ lysis} = 100 \times (\text{experimental release} - \text{spontaneous release})/(\text{total release} - \text{spontaneous release}).$$

For blocking studies, anti-HLA-A2 mAb (gracious gift of Prof. Nagahiro Minato, Department of Immunology and Cell Biology, Kyoto University) was incubated with labeled targets in each well just before adding effector cells. All assays were carried out in triplicate and results are presented as mean ± standard deviation.

RESULTS

Induction of HLA-A2-Restricted and MAGE-3-Derived Peptide-Specific CTLs

To examine the presence of FLWGPRALV-specific CTLs, we compared the power of specific effector induction with the relevant peptide and EVDPIGHLY using autologous DCs as stimulator cells. For induction of effector, we used PBMC and DC from a long survivor with HLA-A2(+) and MAGE-3(+) esophageal cancer. The characteristics of cultured DCs were previously described [4]. They were pulsed with FLWGPRALV or EVDPIGHLY for 60 min at 37°C in AIM-V medium. IL-7 was added to all cultures from the beginning of culture and IL-2 was added 48 hr later. The responder cells were stimulated every 7 days with autologous peptide-pulsed DCs. On day 21, their surface phenotypes and functions were analyzed. The cell population cultured with FLWGPRALV-pulsed DCs highly expressed CD3+ phenotype (82%) and showed CD8+ dominant proportion (77%), while that with EVDPIGHLY-pulsed DCs had low expression of CD3 (54%) and no significant difference between CD4+ cells (18%) and CD8+

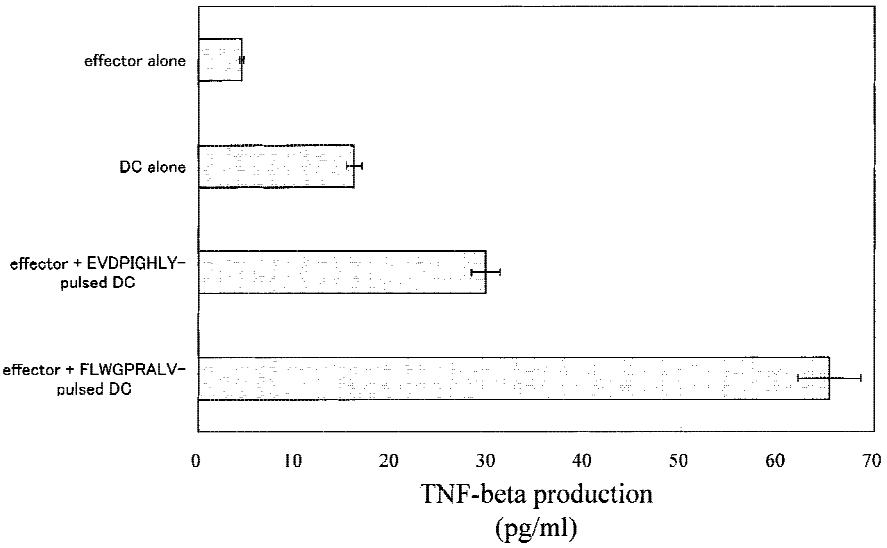

Fig. 3. TNF production using effectors cocultured with FLWGPRALV-pulsed DCs. On day 28, TNF-beta production was measured by colorimetric assay using WEHI 164 clone 13. The bars indicate the standard deviation. As the effectors were restimulated by FLWGPRALV-pulsed DCs, the increase in TNF-beta production was statistically significant, compared with other groups (*t*-test, $P = 0.01$). These are representative data from 3 separate experiments. Effector alone: lymphocytes cultured without DC; DC alone: DCs not cocultured with effector; EVDPIGHLY: HLA-A1-restricted and MAGE-3-gene-derived peptide (amino acids 161–169); FLWGPRALV: HLA-A2-restricted and MAGE-3-gene-derived peptide (amino acids 271–279).

cells (24%), as the representative data from 2 separate experiments.

On day 21, we examined their proliferative responses against the relevant or control peptide-pulsed DCs. The relevant peptide-responding population that had been stimulated with relevant peptide-pulsed DCs increased significantly during 6 days culture (from 0.09 ± 0.02 of which control population without stimulation to 0.23 ± 0.01 in optical density (OD) 560 absorbance of MTT colorimetric assay, *t*-test, $P = 0.01$). However, the numbers of another population stimulated with EVDPIGHLY-pulsed DCs did not change (from 0.09 ± 0.02 to 0.10 ± 0.01 in OD 560 absorbance), as the representative data from 3 separate experiments.

On day 28, we measured the TNF production of these relevant peptide-responding population against the DCs pulsed with or without relevant or control peptide (Fig. 3). They significantly produced TNF-beta (65 ± 3 pg/ml) against relevant peptide-pulsed DCs compared with that (29 ± 2 pg/ml) against control peptide-pulsed DCs ($P = 0.01$).

Specific Cytolytic Activities Against Autologous Tumor Cell Line

The cytolytic activities of these effectors cocultured with relevant peptide-pulsed DCs were assayed in 4 hr Cr-release assay at 5 weeks after the beginning of culture (Figs. 4, 5). They had cytolytic activity against MAGE-3(+) and HLA-A2(+) autologous tumor cell line KYSE-170 and FLWGPRALV-pulsed autologous DCs (% lysis: 17% and 34%, respectively). However, they did not show cytolytic activity against MAGE-3(+) and HLA-A2(−) allogeneic tumor cell line KYSE-520 (% lysis: 1%) (Fig. 4). Moreover, blocking assay using anti-HLA-A2 mAb demonstrated a significant decrease of cytolytic activity against KYSE-170 (from 28% to 5%, $P = 0.01$) and the relevant peptide-pulsed DCs (from 55% to 26%, $P = 0.02$) (Fig. 5). These results showed that FLWGPRALV-specific CTLs were induced by coculture with autologous lymphocytes and relevant peptide-pulsed DCs and they also recognized the autologous tumor cell line, which might have MAGE-3-specific peptide presentation on their HLA-A2 molecules.

DISCUSSION

In this study, we demonstrated that a coculture system with autologous lymphocytes and FLWGPRALV-pulsed autologous DCs could induce the relevant peptide-specific CTLs, and they showed specific cytolytic activity against an autologous esophageal cancer cell line (KYSE-170), which had MAGE-3 mRNA and HLA-A2 molecules.

Recently, several reports have been published about CTL induction using CTL-defined peptides with various APCs and cytokine combination in vitro. Salgaller et al. [13] reported the induction of HLA-A1-restricted and MAGE-1-derived peptide (EADPTGHSY [12])-specific CTL from coculture with tumor infiltrating lymphocytes and EBV-transformed B cells, which was originally defined by MZ2 CTL 82/30, but it was not easy to induce EADPTGHSY-specific CTL in several follow-up trials. Celis et al. [2] reported the induction of MAGE-3-

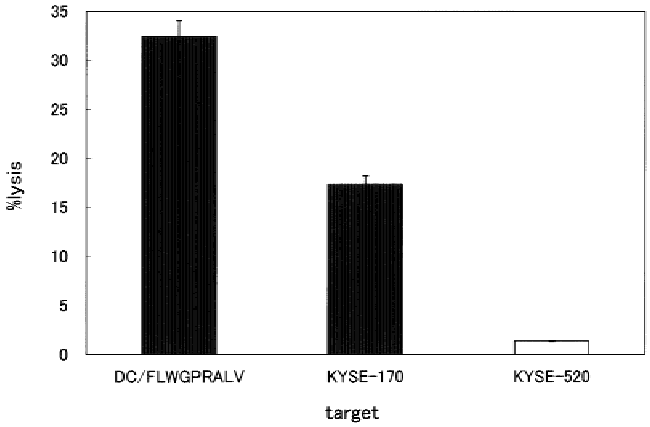

Fig. 4. Cytolytic assay using effectors cocultured with FLWGPRALV-pulsed DCs at 5 weeks after the beginning of culture. The bars indicate the standard deviation. Effectors showed cytolytic activities against DC/FLWGPRALV (*t*-test, $P = 0.01$) and KYSE-170 ($P = 0.03$), but had no cytolytic activity against KYSE-520. DC/FLWGPRALV: FLWGPRALV-pulsed autologous DC; KYSE-170: HLA-A2(+) and MAGE-3(+) autologous tumor cell line; KYSE-520: HLA-A2(−) and MAGE-3(+) allogeneic tumor cell line.

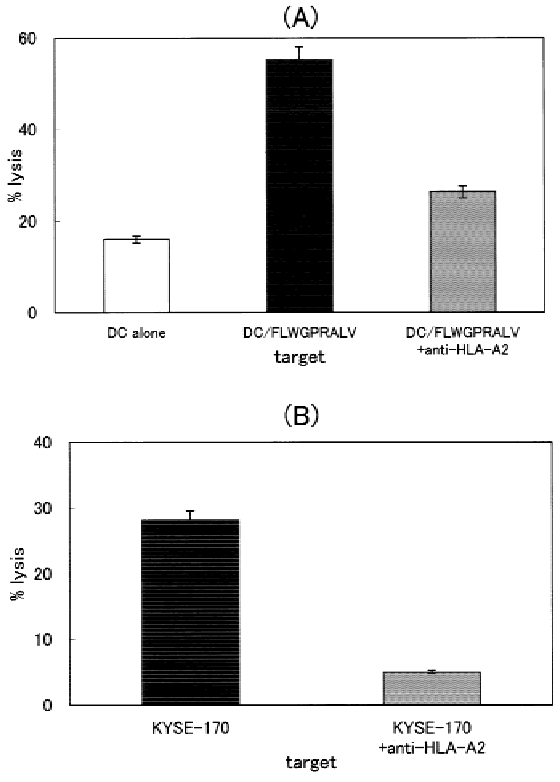

Fig. 5. Blocking assay using anti-HLA-A2 mAb. **(A)** Cytolytic activities against peptide-pulsed DCs blocked by anti-HLA-A2 mAb (*t*-test, $P = 0.01$). Control: cytolytic activities against peptide-unpulsed DCs (16%). **(B)** Cytolytic activities against KYSE-170 blocked by anti-HLA-A2 mAb ($P = 0.02$). The bars indicate the standard deviation.

derived peptide-specific CTL from HLA-A1 healthy donor (EVDPIGHLY) using Staphylococcus Cowan-1 (SAC-1)-activated PBMC as APCs, and van der Bruggen et al. [7] reported the induction of FLWGPRALV-specific CTLs from an HLA-A2 healthy donor using SAC-1-activated PBMC with IL-6 and IL-12 as cytokines. Moreover, using acid-treated PBMC APCs, Valmori et al. [14] reported the induction of FLWGPRALV-specific CTLs from a patient with MAGE-3(+) melanoma. These peptides might be available for target peptides against various cancers with the MAGE gene family.

Among these peptides, FLWGPRALV will be a good candidate for clinical application of peptide-based vaccine against esophageal and gastric cancer because HLA-A2 is a popular allele in Japanese [15] and the MAGE gene family is highly expressed in not only melanoma but also esophageal cancer and gastric cancer [16,17]. In our study, expressions of MAGE-1 and MAGE-3 genes are 88% and 88% in esophageal cancers (11 samples) and also 60% and 45% in gastric cancers (37 samples) (unpublished data).

It is, however, unclear whether major histocompatibility complex (MHC) class I-bound peptide could induce the peptide-specific MHC class I-restricted CTL in vivo. Several recent reports of peptide vaccine against virus or tumor have suggested that cytoplasmic loading methods with professional APCs using virus vector or toxin conjugates could reveal cell-mediated immunity against such peptides in vivo [18].

As professional APCs, DCs are the most powerful APCs to educate naive T cells and induce antigen-specific immunity with MHC in a class II- and class I-restricted manner. Application of DCs as peptide carriers might be more immunogenic and effective for cancer treatment than immunization with peptide alone. Both in the murine tumor system and in the human system, several trials using the combination of DCs and peptide have been attempted and it has been suggested that these methods could induce the peptide-specific CTL and antitumor effect in vivo [19–22].

However, in tumor-bearing hosts or cancer patients, there is controversy concerning the recognition of autologous tumor and/or allogeneic tumor cell lines that had mRNA of CTL-defined antigens. In this context, antigenic peptide presentation by autologous and/or allogeneic tumor cells was the first major hurdle, depending on the surface MHC expression, transporter associated with antigen processing (TAP) function, or amount of relevant antigenic proteins. The second hurdle was the culture method for induction or amplification of peptide-specific CTLs, depending on the stimulator cells (APCs), cytokine combination, and timing of restimulation. The third hurdle was the presence of CTL precursor in the PBMC from which the peptide-specific CTL was induced, de-

pending on the healthy donor, the tumor bearer, or the long survivor. For these reasons, it was difficult to get the generalized concepts for induction of autologous tumor-specific CTL using the CTL-defined peptides.

Using cultured DCs, HLA-A2-restricted and MAGE-3-gene-derived peptide and PBMC of a 10-year survivor with an autologous esophageal cancer cell line that was established from the resected specimen, we showed the presence of peptide-specific CTL which recognized the autologous tumor cell line.

From these points of view, the relevant peptide-pulsed DCs will elicit tumor-specific immune response. For clinical application, it will become a useful immunization method for various types of cancer. We are now cloning this CTL line and determining which T-cell receptor usage is responsible for cytolytic activity.

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